REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2004	Annual (1 May 2003 - 30 Apr 2004)

4. TITLE AND SUBTITLE
Adenovirus-mediated p202 Gene Transfer in Breast Cancer Gene Therapy

5. FUNDING NUMBERS
DAMD17-02-1-0451

6. AUTHOR(S)
Yi Ding, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-Mail: eyiding@mdanderson.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The HIN-200 family are IFN-inducible proteins that share a signature 200-amino acid motif of type *a* and/or *b*. Three human (IFI16, MNDA, and AIM2) and four mouse (p202, p203, p204, and D3) HIN-200 family proteins have been identified. Genes encoding HIN-200 family proteins in both mouse and human are located at chromosome 1q21-23 and form a gene cluster. HIN-200 proteins are primarily nuclear proteins involved in transcriptional regulation of genes important for cell cycle control, differentiation, and apoptosis. Our previous studies have estabnlished that p202 suppressed tumor growth, reduced tumorigenicity, induced apoptosis, and suppressed metastasis and tumor angiogenesis of many human cancer cell lines. The main goal of this project is to study the anti-tumor activity of p202 (aim 1), and the potential application to breast cancer gene therapy (aim 2). Aim 3 is based on our recent discovery of a novel human HIN-200 gene, IFIX (IFN-Inducible protein X). Our preliminary data showed the expression of IFIX is reduced in breast tumor tissues and breast cancer cell lines and that the enforced expression of IFIX in breast cancer cell lines reduces their growth and tumorigenicity. We also demonstrated the treatment efficacy of an IFIX-based gene therapy in an orthotopic breast cancer model. Together, we hypothesize that IFIX functions as a tumor suppressor and may be developed as a therapeutic agent in breast cancer treatment. The new aim will further test the above hypothesis.

14. SUBJECT TERMS
Ad-p202, IFIX, HIN-200 family, breast cancer, gene therapy

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

Award Number: DAMD17-02-1-0451

TITLE: Adenovirus-mediated p202 Gene Transfer in Breast Cancer Gene Therapy

PRINCIPAL INVESTIGATOR: Yi Ding, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: May 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Table of Contents

Introduction

The HIN-200 family are IFN-inducible proteins that share a signature 200-amino acid motif of type *a* and/or *b*. Three human (IFI16, MNDA and AIM2) and four mouse (p202, p203, p204, and D3) HIN-200 family proteins have been identified. (1-4 and see the appendices). Genes encoding HIN-200 family proteins in both mouse and human are located at chromosome 1q21-23 and form a gene cluster (1, 3). HIN-200 proteins are primarily nuclear proteins involved in transcriptional regulation of genes important for cell cycle control, differentiation, and apoptosis (1, 3, 4). Our previous studies have shown that p202 suppressed tumor growth, reduced tumorigenicity, induced apoptosis, and suppressed metastasis and tumor angiogenesis of many human cancer cell lines (5-8).

In search for the potential human ortholog of mouse p202, we recently identified a new member of the human HIN-200 protein family, IFIX (IFN-Inducible protein X). We found that the expression of IFIX is reduced in breast tumor tissues and breast cancer cell lines and that the enforced expression of IFIX in breast cancer cell lines reduces their growth and tumorigenicity. We also demonstrate the treatment efficacy of an IFIX-based gene therapy in an orthotopic breast cancer model. Together, our data suggest that p202 and IFIX functions as a tumor suppressor and may be developed as a therapeutic agent in breast cancer treatment.

Body

A. Objectives

1) Determine the Ad-p202-mediated anti-tumor activities *in vitro*.
2) Determine the Ad-p202-mediated anti-tumor activities *in vivo*.
3) Determine the anti-tumor activity of IFIX.

B. Studies and results

In the past year, we have tested the growth inhibitory activity of Ad-p202 on a panel of breast cancer cell lines. We found Ad-p202 infection resulted in growth inhibition of MDA-MB-453, but had limited effect on other cell lines. It may be due to the limited infection efficiency on these cells. Recently, we isolated IFIX, a p202-like HIN-200 gene in human. However, IFIX is not a human counterpart of p202 since they differ significantly on their protein structure. Most notably, IFIX possesses only one type a 200-amino acid motif but p202 contains both type a and b domain. The N-terminus of IFIX contains a pyrin domain but p202 does not have. We found that IFIX encodes at least 6 isoforms which are likely the results of alternative splicing. Like most of the HIN-200 family members, they are nuclear proteins. IFN-γ treatment suppresses the growth of MCF-7 and MDA-MB-468 breast cancer cells, which correlated with the induction of IFIX. Furthermore, we observed the up-regulation of p21 in IFIX stably expressed MDA-MB-468 and MCF-7 breast cancer cell lines. Most importantly, we found that IFIX expression is reduced in breast tumor tissues and breast cancer cell lines. This observation suggests that IFIX is a novel putative tumor suppressor in breast cancer. To further confirm this result, we found that the IFIX expression is reduced in a panel of commercially available breast carcinoma tissues. Like p202, our data show that IFIX possesses tumor suppressor activity in breast cancer and suggest that IFIX may be used as a therapeutic agent in cancer treatment.

The progress of each objective is discussed below:

Objective 1: Determine the Ad-p202-mediated anti-tumor activities *in vitro*.

1) In addition to MDA-MB-468 cells, we found Ad-p202 can inhibit MDA-MB-453 cell growth (Fig 1). However, we observed limited inhibitory effect on other breast cancer cell lines, e.g., MDA-MB-231 and MDA-MB-435. It is possible due to low infection efficiency in these cells.

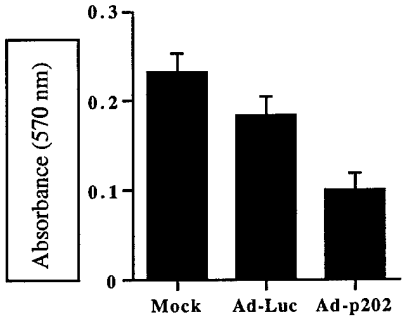


Fig.1 Ad-p202 Infection inhibits cells proliferation. MDA-MB-453 cells were infected with Ad-Luc or Ad-p202 at a MOI of 30. Cell growth was monitored after infection for 48 h by MTT assay.

2) A recent report has identified the NF-*k*B-interacting domain on p202 (9). Now we are in a position to use this information to generate expression vectors that express the NF-*k*B-binding domain and test whether this domain alone is able to sensitize cells to TNF-α -induced apoptosis.

Objective 2: Determine the Ad-p202-mediated anti-tumor activities *in vivo*.

1) Determination of the efficacy of a combined treatment of Ad-p202 with TNF-α in an orthotopic breast cancer animal model: in progress.

Objective 3: Determine the anti-tumor activity of IFIX.

1) We cloned IFIX, a novel human HIN-200 gene. We found that there are at least six IFIX isoforms (IFIX α1, α2, β1, β2, γ1 and γ2). Like most of the HIN-200 family members, they are nuclear proteins (please see the attached reprint, Fig 1, Fig. 2, Fig. 4 d and Fig. 8).
2) IFN-γ treatment suppresses the growth of MCF-7 and MDA-MB-468 breast cancer cells, which correlated with the induction of IFIX (please see the attached reprint, Fig. 5a)
3) We found IFIX expression is reduced in breast tumors and breast cancer cell lines. (please see the attached reprint, Fig. 4 a, b, c).
4) IFIX-α 1 up-regulates p21, a key cyclin-dependent kinase inhibitor, leading to cell cycle arrest. (please see the attached reprint, Fig. 7).

Key Research Accomplishments:

1) Infection of Ad-p202 inhibits MDA-MB-453 cells growth.
2) IFN-γ treatment suppresses the growth of MCF-7 and MDA-MB-468 breast cancer cells, which correlated with the induction of IFIX.
3) IFIX-α 1 up-regulate p21.
4) IFIX is down-regulated in breast tumors.

Reportable Outcomes:

1) **Yi Ding**, Li Wang, Li-Kuo Su, Jennifer A. Frey, Ruping Shao, Kelly K. Hunt, and Duen-Hwa Yan. Anti-tumor activity of IFIX, a novel interferon-inducible HIN-200 gene, in breast cancer. (Oncogene in press)

2) US Patent pending
Title: IFIX, a novel HIN-200 protein, for cancer therapy.
Inventors: Duen-Hwa Yan, **Yi Ding**, Li Wang, and Mien-Chie Hung

Training accomplishments:

1) A poster presentation (Abstract No. 3511) at 95th AACR meeting, Orlando, FL.
2) A poster presentation at Trainee Recognition 2004, U. T. M. D. Anderson Cancer center.
3) Attended department seminars (12:00 am-1:00pm, Wednesday), institutional seminars (12:00 am-1:00pm, Friday) and other seminars.

Conclusions:

Our studies show p202, an IFN-inducible protein, possesses pro-apoptotic and anti-tumor activities *in vitro* and *in vivo*. We found that Ad-p202 infection resulted the cell growth inhibition of another breast cancer cell line, MDA-MB-453. We will continue to investigate whether the combination of p202 and these therapeutic agents, TNF-α, CDDP, Taxol, might achieve synergistic (or additive) therapeutic efficacy *in vivo*.

We cloned a novel HIN-200 family member, IFIX. Six different alternatively spliced forms of mRNA are transcribed from the IFIX gene, which are predicted to encode six different isoforms of IFIX proteins (IFIX α1, α2, β1, β2, γ1 and γ2). Like most of the Hin-200 proteins, they are nuclear proteins. Previously, we have shown that the expression of IFIX is reduced in breast tumor tissues and breast cancer cell lines and that the enforced expression of IFIX in breast cancer cell lines reduces their growth and tumorigenicity. We also demonstrated the treatment efficacy of an IFIX-based gene therapy in an orthotopic breast cancer model. Here we further confirmed the down-regulation of IFIX in breast tumors, by examine the expression of IFIX using a panel of commercially available human cDNAs. We also found that IFIX-α 1 up-regulate p21, which leads to the reduction of the kinase activity of both Cdk2 and p34^{cdc2}, and the cell cycle arrest at G1 (MCF-7 cells) or G2/M phase (MDA-MB-468 cells). Together, our data suggest that IFIX possesses tumor suppressor activity in breast cancer and that IFIX may be used as a therapeutic agent in breast cancer treatment.

References:

1. Lengyel, P., Choubey, D., Li, S.-J., & Datta, B. (1995) *Semi Virol* **6,** 203-213.
2. Landolfo, S., Gariglio, M., Gribaudo, G. & Lembo, D. (1998) *Biochimie* **80,** 721-728.
3. Johnstone, R. W. & Trapani, J. A. (1999) *Mol Cell Biol* **19,** 5833-5838.
4. Choubey, D. (2000) *J Biol Regul Homeost Agents* **14,** 187-192.
5. Yan, D.-H., Wen, Y., Spohn, B., Choubey, D., Gutterman, J. U., & Hung, M.-C. (1999) Oncogene**18**, 807-811.
6. Wen, Y., Yan, D.-H., Wang, B., Spohn, B., Ding, Y., Shao, R., Zhou, Y., Xie, K. &Hung, M-C. (2001) Cancer Res **61**, 7142-7147.
7. Wen, Y., Yan, D. H., Spohn, B., Deng, J., Lin, S. Y. & Hung, M. C. (2000) Cancer Res **60**, 42-46.

8. Ding, Y., Wen, Y., Spohn, B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., Hung, M.-C. & Yan, D.-H. (2002) *Clin Cancer Res* **8,** 3290-3297.
9. Xian-YongMa, Hong Wang, Bo Ding, Haihong Zhong, Sankar Ghosh, and Peter Lengyel. (2003) J.Biol.Chem. **278**, 23008-23019.

Appendices

Reprint

Yi Ding, Li Wang, Li-Kuo Su, Jennifer A. Frey, Ruping Shao, Kelly K. Hunt, and Duen-Hwa Yan. Anti-tumor activity of IFIX, a novel interferon-inducible HIN-200 gene, in breast cancer. (Oncogene in press)

Oncogene (2004), 1–11

www.nature.com/onc

ORIGINAL PAPER

Antitumor activity of IFIX, a novel interferon-inducible HIN-200 gene, in breast cancer

Yi Ding[1], Li Wang[1], Li-Kuo Su[1,3], Jennifer A Frey[1,3], Ruping Shao[1], Kelly K Hunt[2] and Duen-Hwa Yan*[,1,2,3]

[1]*Department of Molecular and Cellular Oncology, The University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA;* [2]*Department of Surgical Oncology, The University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA;* [3]*The University of Texas Graduate School of Biomedical Sciences at Houston, USA*

We identified IFIX as a new member of the hematopoietic interferon (IFN)-inducible nuclear protein with the 200-amino-acid repeat (HIN-200) family. Six different alternatively spliced forms of mRNA are transcribed from the IFIX gene, which are predicted to encode six different isoforms of IFIX proteins (IFIXα1, α2, β1, β2, γ1, and γ2). The IFIX proteins are primarily localized in the nucleus. They share a common N-terminal region that contains a predicted pyrin domain and a putative nuclear localization signal. Unlike IFIXα and IFIXβ, IFIXγ isoforms do not have the 200-amino-acid signature motif. Interestingly, the expression of IFIX was reduced in most human breast tumors and breast cancer cell lines. Expression of IFIXα1, the longest isoform of IFIX, in human breast cancer cell lines reduced their anchorage-dependent and -independent growth *in vitro* and tumorigenicity in nude mice. Moreover, a liposome-mediated IFIXα1 gene transfer suppressed the growth of already-formed tumors in a breast cancer xenograft model. IFIXα1 appears to suppress the growth of breast cancer cells in a pRB- and p53-independent manner by increasing the expression of the cyclin-dependent kinase inhibitor $p21^{CIP1}$, which leads to the reduction of the kinase activity of both Cdk2 and $p34^{Cdc2}$. Together, our results show that IFIXα1 possesses a tumor-suppressor activity and suggest IFIXα1 may be used as a therapeutic agent in cancer treatment.

Oncogene advance online publication, 3 May 2004; doi:10.1038/sj.onc.1207592

Keywords: IFIX; interferon; HIN-200; $p21^{CIP1}$; breast cancer

*Correspondence: D-H Yan, Department of Molecular and Cellular Oncology, The University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, USA. Tel: 713-792-3677, Fax: 713-794-0209; E-mail: dyan@mdanderson.org
Received 16 July 2003; revised 30 October 2003; accepted 29 January 2004

Introduction

The interferon (IFN) family of cytokines is known for its growth-inhibitory activity, which plays an important role in IFN-mediated antitumor activity (Kimchi *et al.*, 1988). Proteins induced by IFN are thought to play important roles in mediating the antitumor activity of IFN (Lengyel, 1993). HIN-200 family proteins are IFN-inducible proteins that share a 200-amino-acid signature motif of type *a* and/or *b*. Three human (IFI16, MNDA, and AIM2) and five mouse (p202*a*, p202*b*, p203, p204, and D3) HIN-200 family proteins have been identified (Johnstone and Trapani, 1999; Choubey, 2000). Genes encoding HIN-200 family proteins are located at chromosome 1q21–23 and form a gene cluster in both mouse and human (Johnstone and Trapani, 1999). HIN-200 proteins are primarily nuclear proteins involved in the transcriptional regulation of genes important for cell cycle control, differentiation, and apoptosis (Johnstone and Trapani, 1999; Choubey, 2000). The antitumor activity of HIN-200 proteins has been demonstrated. In particular, we have shown that p202*a* suppressed tumor growth, reduced tumorigenicity, induced apoptosis, and suppressed metastasis and tumor angiogenesis of human cancer cells (Wen *et al.*, 2000, 2001; Ding *et al.*, 2002). The amino-acid sequence identity between the three human HIN-200 family proteins and the mouse p202*a* are 40% or less, thus none of these human proteins appears to be the ortholog of p202*a*. In a search for potential new human HIN-200 proteins, we have identified a new member of the human HIN-200 protein family, IFN-inducible protein X (IFIX). There are at least six IFIX isoforms encoded by alternatively spliced mRNAs (Figures 1a and 2a). We show here that the mRNA level of IFIX is reduced in breast tumor tissues and breast cancer cell lines and that expression of IFIXα1 reduces growth and tumorigenicity of breast cancer cell lines that have undetectable levels of IFIX expression. We also demonstrate the treatment efficacy of an IFIXα1-based gene therapy in an orthotopic breast cancer model. Together, our data suggest that IFIXα1 functions as a tumor suppressor and may be developed as a therapeutic agent in breast cancer treatment.

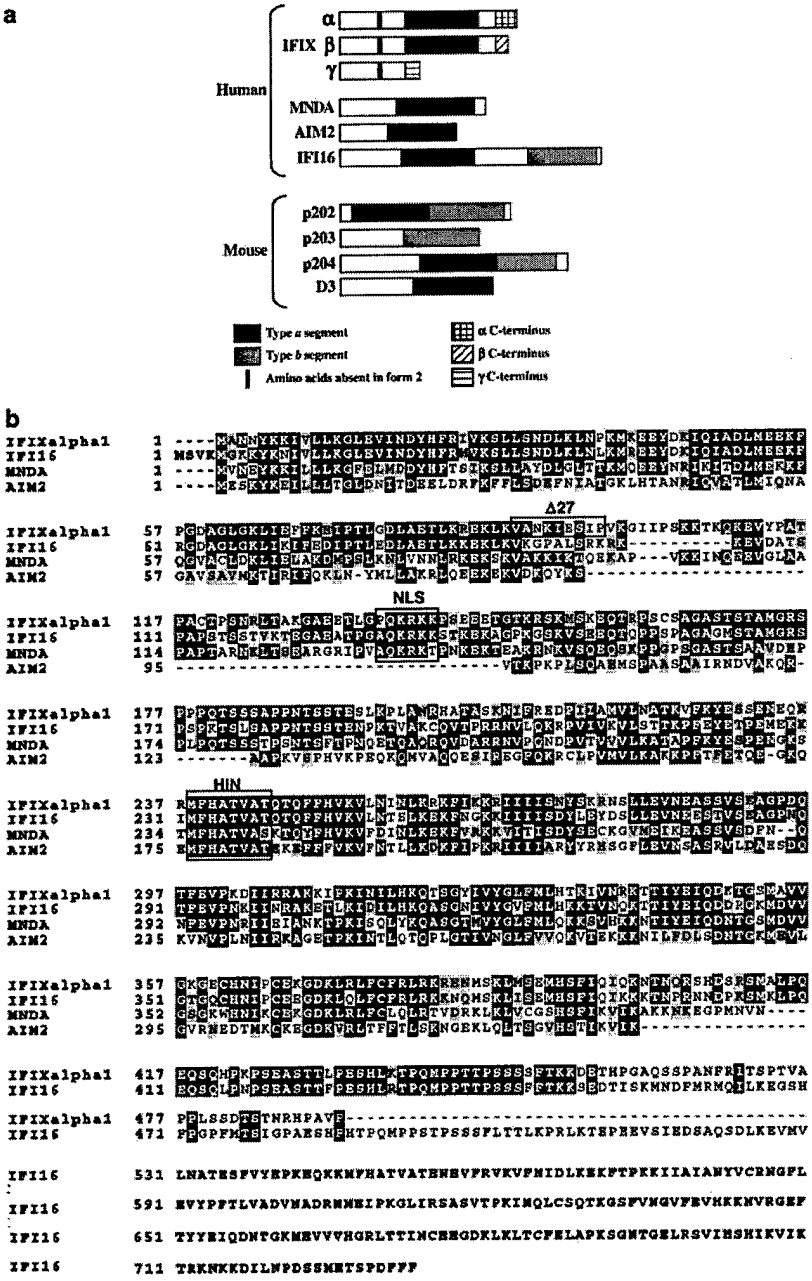


Figure 1 Characterization of IFIX. (a) Structural comparison among HIN-200 proteins. Black and gray bars indicate the type *a* and type *b* 200-amino-acid signature motifs of HIN-200 proteins, respectively. Different patterns of the C-terminus of IFIX isoforms indicate different amino-acid sequences of their C-terminal S/T/P-rich domains. The nine amino acids absent in $\alpha 2$, $\beta 2$, and $\gamma 2$ isoforms are indicated. (b) Amino-acid sequence comparison among human HIN-200 proteins. Amino acids identical or similar in at least two sequences are highlighted with black or gray background, respectively. Dashes indicate gaps introduced in the sequence to obtain the best alignment. The putative nuclear localization signal (NLS) and the nine-amino-acid deletion (Δ27) are indicated. The common MFHATVAT (HIN) in the 200-amino-acid signature motif of the human HIN-200 proteins is indicated

Results

IFIX is a novel human HIN-200 gene

To identify potential new human HIN-200 proteins, we used the amino-acid sequence of p202*a* to query human-specific nr, est, and htgs databases at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) by using the tblastn protocol. These queries identified a new gene IFIX in addition to three previously known human HIN-200 family members, MNDA, IFI16, and AIM2. The IFIX gene is located between MNDA and IFI16 at chromosome 1q21–23.

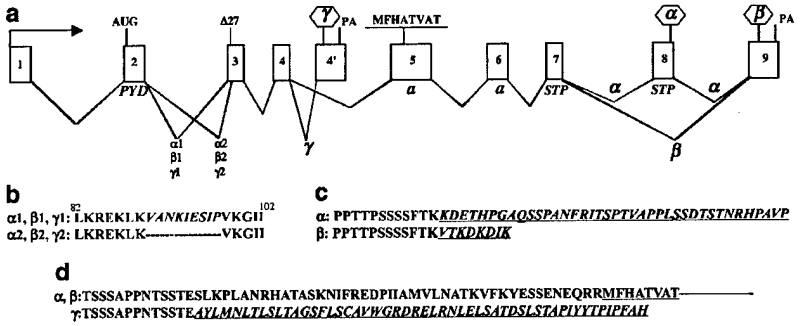


Figure 2 Structures of IFIX isoforms. *a*. Schematics of the *IFIX* gene. Exons of the *IFIX* gene are shown as open boxes and the exon numbers are indicated, the size of exons and introns are not drawn to scale. Alternative splicing events that result in various IFIX isoforms are indicated. Arrow indicates a putative transcriptional start site determined by 5′ rapid amplification of cDNA ends. AUG: the translation initiation codon; *PYD*: the putative pyrin domain; Δ27: the 27 bp absent in the α2, β2, and γ2 isoforms; the hexagons: stop codons of the α, β, and γ isoforms; PA: polyadenylation signal; MFHATVAT: an amino-acid sequence shared among HIN-200 proteins. *a*: the type *a* 200-amino-acid repeat; *STP*: serine/threonine/proline-rich region. The predicted size of each isoform is as follows: α1, 492 aa, α2, 483 aa, β1, 461 aa, β2, 452 aa, γ1, 246 aa, and γ2, 237 aa. *b*: The nine amino acids (VANKIESIP) absent in isoforms α2, β2, and γ2 are italicized. *c*: The C-terminal amino-acid sequences of isoforms α and β. Amino acids different between isoforms α and β are italicized and underlined. *d*: The unique C-terminal amino-acid sequence of the γ isoforms. Amino acids of γ isoforms different from isoforms α and β are italicized and underlined. The MFHATVAT signature motif of HIN-200 proteins in α and β isoforms is underlined

The IFIX cDNAs were obtained by RT–PCR using total RNA isolated from IFN-α-treated Daudi cells. Each cDNA clone was confirmed by DNA sequencing. We identified at least six IFIX isoforms (α1, α2, β1, β2, γ1, and γ2) that are homologous to other human and mouse HIN-200 proteins (Figure 1a). The identity between the amino-acid sequence of IFIXα1 and other members of human HIN-200 family is: IFI16, 67%; MNDA, 53%; and AIM2, 31% (Figure 1b). IFIX is unlikely to be the human ortholog of p202*a* because the similarity between the amino-acid sequences of IFIX and p202*a* is only limited to the type *a* 200-amino-acid signature motif (Figure 1a). The IFIX isoforms are likely derived from the alternative splicing based on the comparison between the cDNA sequences and the genomic sequence (Figure 2a). IFIXα2, β2, and γ2 have a deletion of identical nine amino acids, that is, ^{89}VANKIESIP (resulting from alternative splicing), in their N-terminal region when compared to IFIXα1, β1, and γ1 (Figure 2b). The α and β isoforms contain a type *a* 200-amino-acid signature motif of HIN-200 proteins whereas γ isoforms do not have this motif. The C-termini of α, β, and γ isoforms are diverse due to alternative splicing (Figure 2c and d). The IFIX isoforms share a common N-terminal region, which contains a predicted pyrin domain (amino acids 3–88), a protein–protein interaction module involved in apoptotic and inflammatory signaling pathways (Fairbrother *et al*., 2001; Martinon *et al*., 2001; Staub *et al*., 2001). In addition, the N-terminal region also contains a putative nuclear localization signal, ^{134}LGPQKRKK (Figure 1b, Dawson and Trapani, 1995). Consistent with that prediction, we found the stably transfected IFIXα1 as well as the EGFP-tagged IFIXα1, β1, and γ1 fusion proteins are localized in the nucleus (Figure 8a and b). Interestingly, while IFIXα1 and IFIXβ1 are primarily localized in the nucleoplasm, IFIXγ1 forms a speckled nuclear pattern (Figure 8b). This observation suggests that, like most of the HIN-200 proteins, the IFIX proteins are primarily nuclear proteins. Consistent with the notion that HIN-200 genes are IFN inducible, the IFIX mRNA levels are characteristically induced by IFN-α and IFN-γ in several human cancer cell lines of hematopoietic origin (Figure 3a). A tissue distribution study showed that IFIX mRNA (~2.4 kb) is readily detected in the spleen, lymph node, and peripheral blood leukocyte, but to a less extent in thymus, bone marrow, and fetal liver (Figure 3b). No detectable level of IFIX mRNA was found in adult brain, heart, skeletal muscle, colon, kidney, liver, small intestine, placenta, and lung (data not shown). These results suggest that IFIX expression may be involved in immune response.

IFIX is downregulated in human breast cancers

AIM2 has been suggested to play a role in tumorigenesis (De Young *et al*., 1997). We therefore investigated whether the expression of IFIX was altered in human cancers. We examined the expression of IFIX using a panel of commercially available human cDNAs derived from 12 normal breast tissues (normal) and 12 breast carcinoma tissues (tumor) (Origene Technologies, Inc. Rockville, MD, USA see Materials and methods) by PCR using primers specific to IFIXα. As shown in Figure 4a, IFIXα expression is detectable in 10 out of 12 normal breast tissue samples. In contrast, only two out of 12 breast carcinoma tissues have detectable IFIXα expression. This result suggests IFIX is downregulated in breast tumors. We further tested this correlation in the matched normal and tumor tissues collected from five breast cancer patients by RT–PCR using primers specific to IFIXα. The expression of IFIXα was detected in all tissues examined however, the level of IFIXα in tumor tissue was lower than that in the normal tissue of each patient (Figure 4b). We also examined the

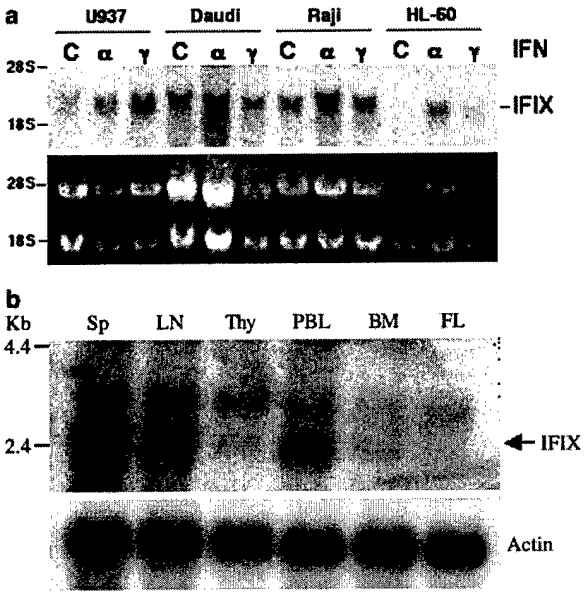


Figure 3 (**a**) Induction of IFIX expression by IFN. The IFIX mRNA in the indicated cell lines without treatment (c) or treated with 100 U/ml of IFNα (α) or IFNγ (γ) was detected by Northern blot analysis. The 18S and 28S rRNAs serve as loading controls. (**b**) IFIX expresses mainly in the secondary lymphoid organs. The Multiple Tissue Northern blot (BD Biosciences) was hybridized with an IFIXα1 cDNA probe. The IFIX mRNA (IFIX) band is indicated. The actin mRNA served as the loading control. Sp: spleen; LN: lymph node; PBL: peripheral blood leukocyte; Thy: thymus; BM: bone marrow; FL: fetal liver. The molecular weight markers (Kb) are indicated

expression of IFIX in a panel of human breast epithelial cell lines. IFIX expression was detected in all the three nontumorigenic cell lines. In contrast, seven out of nine breast cancer cell lines did not express detectable IFIX (Figure 4c and 7a). These data show that the expression of IFIX is reduced in breast cancer and suggest IFIX may function as a tumor suppressor. To determine the identity of IFIX isoforms in the IFIX-expressing cell lines, we performed RT–PCR using specific primers for these isoforms. As shown in Figure 4d (top and middle panels), the IFIXα, β, and γ isoforms are present in these cell lines, although the 27-bp deletion in the 'form 2' isoforms cannot be distinguished at this gel resolution.

To further determine the presence of the 'form 2' isoforms, we designed primers that flank the Δ27 region (Figure 1b and 2a) followed by RT–PCR. Consistent with the fact that the 'form 2' isoforms were isolated from Daudi cells, the expression of 'form 2' isoforms is detectable in Daudi cells, but the expression levels are much lower than that of the 'form 1' isoforms with the 27-bp region (Figure 4d, bottom panel). However, under

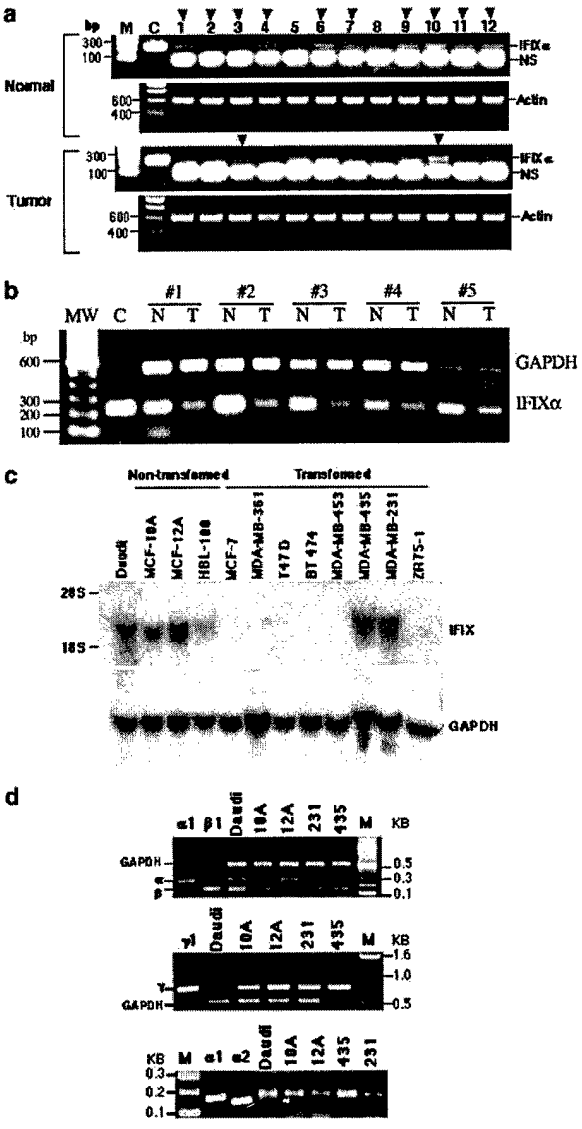


Figure 4 Reduced expression of IFIX in human breast tumor and breast cancer cell lines. (**a**) Reduced expression of IFIX in human breast tumors. The commercially available human cDNAs derived from 12 normal breast tissues (normal) and 12 breast carcinoma tissues (tumor) (Origene Technologies, Inc.) were analysed for IFIX expression by PCR using primers specific to IFIXα. The IFIXα1 cDNA was used as a control (C). Molecular weight markers (M) are indicated. The IFIXα and β-actin specific bands are indicated. NS: nonspecific PCR products. Samples positive for IFIXα expression are indicated by solid triangles. (**b**) Reduction of IFIXα mRNA levels in breast cancer. The IFIXα mRNA levels in normal breast (N) and breast cancer (T) tissues from five breast cancer patients were determined by RT–PCR using primers specific to IFIXα. C, an IFIXα1 cDNA clone used as the template in PCR. RT–PCR of GAPDH was used as a control for the RNA quality and quantity. (**c**) Reduction of IFIX mRNA levels in breast cancer cell lines. IFIX mRNA in 20 μg of total RNA isolated from indicated cell lines was determined using Northern blot analysis. GAPDH mRNA on the same blot was subsequently detected to serve as an RNA-loading control. (**d**) The presence of IFIX isoforms in the IFIX-expressing cell lines. RT–PCR was performed using primers specific for IFIXα, β, (top panel) or γ (middle panel), and the 'form 2' (indicated by an arrowhead, bottom panel) in Daudi, MCF-10A (10A), MCF-12A (12A), MDA-MB-231 (231), and MDA-MB-435 (435). The IFIXα1, α2, β1, and γ1 cDNAs were used as controls

our experimental conditions, the 'form 2' isoforms appeared to be not expressed or undetectable in other IFIX-expressing cell lines.

IFIX α1 suppresses breast cancer cell growth and tumorigenicity

As the first step to investigate the potential role of IFIX in tumor suppression in breast cancer, we use IFIXα1 for subsequent studies because it is the longest isoform of IFIX and possesses the most structural features among IFIX isoforms (Figure 1a and 1b). IFIXα1 is predicted to contain 492 amino acids with an apparent molecular weight of ~53 kDa. To investigate the possible tumor-suppressor function of IFIXα1, we employed two human breast cancer cell lines, MCF-7 and MDA-MB-468, which express very low levels of endogenous IFIX (Figure 4c, 5a, and 7b). Consistent with a previous report (Gooch *et al.*, 2000), we showed that IFN-γ treatment suppressed the growth of these breast cancer cells (Figure 5a, top panel), which correlated with the induction of IFIX (Figure 5a, bottom panel). To determine whether IFIX could suppress cell growth, we stably expressed IFIXα1 in MDA-MB-468 and MCF-7 cells (Figure 5b). Examination of the growth rates of the control cell lines (parental (P) and empty vector stable cell line (V)) and two independent IFIXα1-expressing cell lines (X-1 and X-2) derived from MDA-MB-468 and MCF-7 cells showed that the expression of IFIXα1 reduced the growth of breast cancer cells (Figure 5c). The soft agar assay was used to determine the effect of IFIXα1 on the *in vitro* transformation property. As shown in Figure 5d, the number of foci of IFIXα1-expressing derivatives (X-1 and X-2) was reduced as compared with the control cell lines (P and V). This result indicated that IFIXα1 suppressed the transformation phenotype of breast cancer cells and predicted a loss of tumorigenicity of IFIXα1-expressing breast cancer cells. To test that possibility, the tumorigenicity of the IFIXα1-expressing derivative 468-X-2 was then investigated by implanting 468-X-2 and the control, MDA-MB-468 cells into the mammary fat pad (MFP) of 6-week-old female nude mice. As shown in Figure 5e, while MDA-MB-468 cells (P) are highly tumorigenic, the tumorigenicity of 468-X-2 cells (X) is significantly reduced. Given that IFN is known to mediate growth inhibition and tumor suppression in breast cancer (Zhang *et al.*, 1996; Coradini *et al.*, 1998; Gooch *et al.*, 2000), our data suggest that IFIXα1 is a major mediator of the tumor-suppressor activity of IFN.

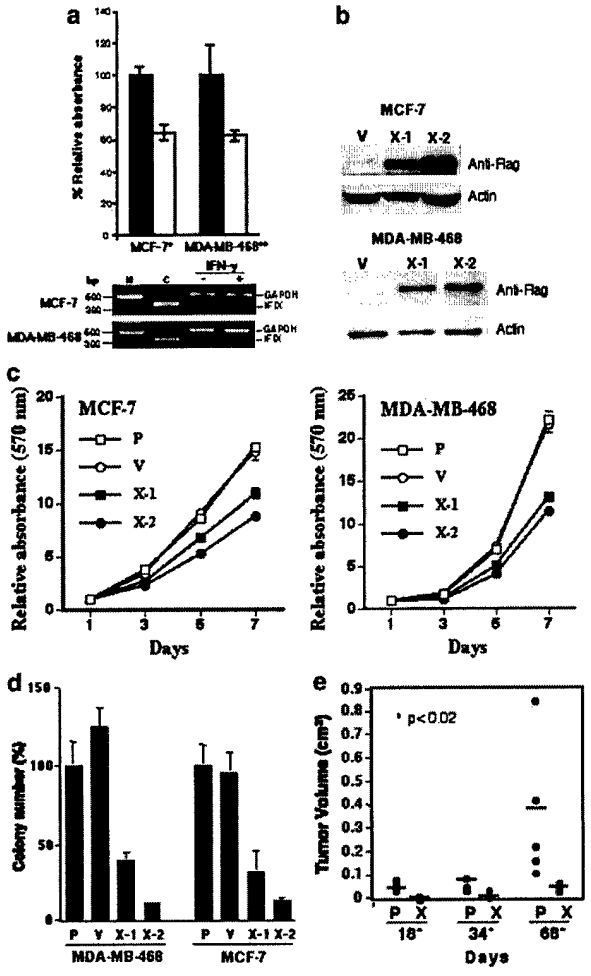


Figure 5 Suppression of the growth and tumorigenicity of breast cancer cells by IFIXα1. (**a**) IFN-γ induces IFIX expression in breast cancer cells. Top panel: MCF-7 and MDA-MB-468 cells were treated with (open bars) or without (solid bars) IFN-γ (1000 U/ml) in DMEM/F12 media containing 0.25% fetal calf serum for 48 h. The growth of the cells was measured by MTT assay. The experiment was run in triplicate and represented as the mean ± s.d. The asterisks represent the statistically significant differences due to the IFN-γ treatment. $*P<0.0005$, $**P<0.036$. Bottom panel: Total RNAs isolated from MCF-7 and MDA-MB-468 cells treated with or without IFN-γ (1000 U/ml) under the same condition as described above were analysed for IFIX expression by RT–PCR. GAPDH was used as an internal control. The IFIXα1 cDNA was used as a specificity control (C). Molecular weight markers (M) are indicated. (**b**) Expression of exogenous IFIXα1 in breast cancer cell lines. The Flag-tagged IFIXα1 in lysates from clones expressing Flag-tagged IFIXα1 (X-1 and X-2) and the empty vector (pCMV-Tag2B) (V) control clones were detected by Western blot using an anti-Flag antibody. The actin protein levels serve as loading controls. (**c**) Reduced growth rates in IFIXα1 stable cell lines. The growth rate of IFIXα1-expressing clones (X-1 and X-2), the parental (P) and the empty vector (V) control clones was measured by MTT assay. Each measurement was made in quadruplicate. The relative absorbance at 570 nm was determined by setting the absorbance on day 1 at 1. The range of variation at some data points is too small to be seen. (**d**) Suppression of *in vitro* transformation by IFIX. The parental (P), the empty vector (V), or IFIXα1 stable cell lines (X-1 and X-2) derived from MDA-MB-468 or MCF-7 cells were seeded in soft agar and the colony number was scored at 3 weeks after seeding. The relative colony numbers of IFIXα1-expressing clones are compared with that of their parental cells (100%). (**e**) Suppression of tumorigenicity by IFIXα1. 468-X-2 and the control, MDA-MB-468 (P) cells were implanted into the MFP of 6-week-old female nude mice at two sites per mouse, three mice per group. The actual size of each tumor at the indicated time points after implantation is presented. Horizontal bars indicate the average tumor size. *t*-test: $*P<0.02$

IFIXα1 treatment results in therapeutic efficacy

To rule out the possibility that the reduced tumorigenicity of 468-X-2 was due to clonal variation (Figure 5e) and to test whether IFIXα1 could suppress breast tumor growth, we performed a preclinical gene therapy experiment using an orthotopic breast cancer xenograft model. Female nude mice were inoculated with MDA-MB-468 cells into their MFP and tumors were allowed to grow to 0.5 cm in diameter. Tumors were then injected with the liposome SN2 complexed with either an IFIXα1-expression vector (CMV-IFIXα1) or an empty vector (pCMV-Tag2B). SN2 was selected as the gene delivery system because it is a nonviral, stable liposome-forming cationic lipid formulation and has been proven to be highly efficient in gene delivery (Zou *et al.*, 2002). As shown in Figure 6, the CMV-IFIXα1/SN2 (X) treatment yielded significant antitumor activity as compared to the pCMV-Tag2B/SN2 (V) treatment. This observation suggests that the reduced tumorigenicity seen in 468-X-2 (Figure 5e) is likely caused by IFIX expression and not by clonal variation. More importantly, this result indicates that IFIXα1 possesses antitumor activity and shows the feasibility of an IFIX-based gene therapy for breast cancer treatment.

IFIXα1 upregulates p21^{cip1}

IFN-induced growth arrest is known to be associated with an elevated level of the cyclin-dependent kinase inhibitor (CKI) p21^{CIP1} (Naldini *et al.*, 2002; Zhou *et al.*, 2002). As the expression of IFIXα1 is induced by IFN (Figure 3a and 5a), we therefore investigated the mRNA and protein levels of p21^{CIP1} in IFIXα1 stable transfectants. As shown in Figure 7a and b, both p21^{CIP1} protein and mRNA levels are upregulated in IFIXα1 stable cell lines (X-1 and X-2) as compared with the control cell lines (P and V). However, there are no detectable changes in the expression of other CKIs, such as p27^{KIP1}, p57^{KIP2}, and p16^{INK4a} in IFIXα1-expressing derivatives (data not shown). Since p21^{CIP1} is a universal CKI, upregulation of p21^{CIP1} should inactivate the kinase activity of Cdk2 in IFIXα1 stable cells. Therefore, we used an immunocomplex kinase assay to determine the Cdk2 kinase activity in IFIXα1 stable cell lines and control cells. As expected, Cdk2 activity is reduced in 468-X-2 and MCF-X-2 as compared to their respective parental cells (Figure 7c). Western blot indicates that there are comparable amounts of Cdk2 protein used in the kinase assay. However, since MDA-MB-468 cells lack pRB and express mutant p53 (Yin *et al.*, 2001), inhibition of MDA-MB-468 cell growth by IFIXα1 cannot simply be attributed to the inactivation of Cdk2 leading to G1/S-phase arrest (MacLachlan *et al.*, 1995). One possible explanation was that p21^{CIP1} also inhibits p34^{Cdc2}, a G2/M-phase Cdk (Yu *et al.*, 1998). We examined the p34^{Cdc2} kinase activity in IFIXα1-expressing MDA-MB-468 cells and found that it was much lower than that of the control cells (Figure 7c). This result suggests that inactivation of p34^{Cdc2} may contribute to the IFIXα1-mediated growth inhibition in MDA-MB-468 cells. To further confirm this observation, we performed a flow cytometry analysis to determine any changes in the cell cycle distributions caused by the expression of IFIX. As shown in Figure 7d, a significant G1-phase accumulation and S-phase reduction was observed in MCF-X-2 cells. In contrast, 468-X-2 cells exhibited a significant S- and G2/M-phase accumulation. This observation not only provides an explanation for the slower growth rate of IFIX stable cell lines (Figure 5c) but also correlates with the inactivation of Cdk2, leading to G1-phase accumulation in MCF-7 in which pRB/E2F pathway is intact and the inactivation of p34^{Cdc2}, resulting in a blockage of G2/M-phase entry in MDA-MB-468 in which pRB/E2F pathway is defective. Together, the data suggest that the p53/pRB-independent p21^{CIP1} upregulation contributes to IFIXα1-mediated antitumor activity in breast cancer cells. To test the ability of different IFIX isoforms to induce p21^{CIP1}, we transiently transfected MCF-7 cells with the plasmids encoding EGFP-tagged IFIXα1, β1, or γ1 fusion protein followed by immunostaining with the p21^{CIP1}-specific antibody. As shown in Figure 8b, the expression of IFIXα1 or β1 mainly coincides with the expression of p21^{CIP1} in the nucleus (64 and 52%, respectively). In contrast, like the empty vector (EGFP) control, the expression of IFIXγ1 has little effect on the expression of p21^{CIP1} (0.95 and 2%, respectively). Together with a unique speckled nuclear pattern, our observations indicate that IFIXγ1 may function differently from IFIXα1/β1, and it also suggests that the

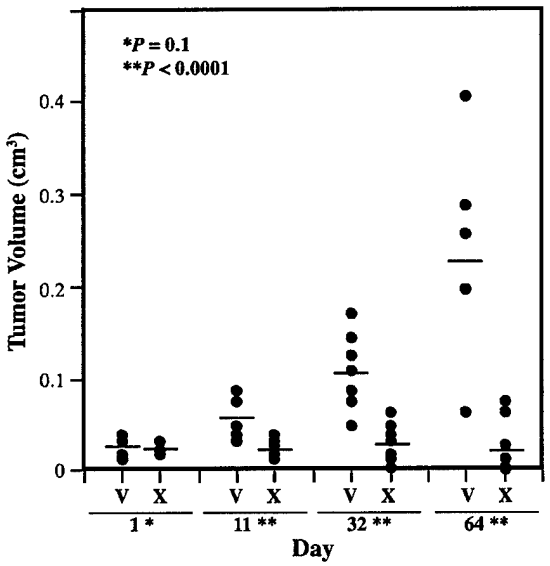
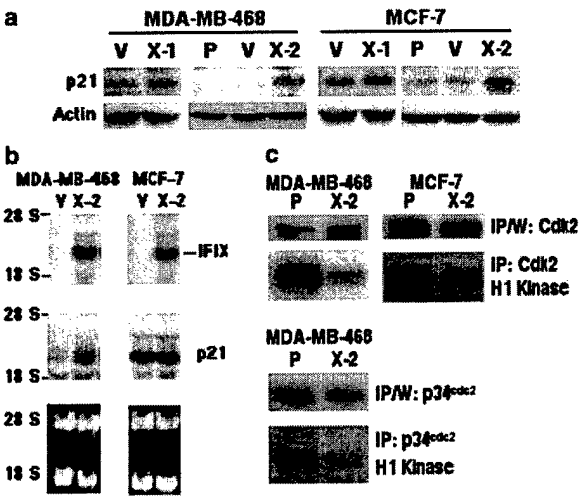


Figure 6 The antitumor effect of IFIXα1/SN2 liposome treatment in an orthotopic breast cancer xenograft model. Orthotopic breast tumors were established by inoculating MDA-MB-468 cells into the MFP of nude mice and the treatments began at tumors about 0.5 cm in diameter. Tumors were treated twice weekly with SN2 mixed with either CMV-IFIXα1 (X) or pCMV-Tag2B (V). The actual size of each tumor at the indicated time points after treatments begun is presented. Horizontal bars indicate the average tumor size. *t*-test: $*P=0.1$, $**P<0.0001$



d

		G1 (%)	S (%)	G2/M (%)
MCF-7	V	46.35±1.35	45.70±0.30	7.95±1.05
	X-2	68.35±2.45	24.40±2.20	7.25±0.25
MDA-MB-468	V	54.25±3.95	30.50±3.10	15.25±0.85
	X-2	34.35±1.35	43.65±0.55	22.00±1.90

Figure 7 Upregulation of $p21^{CIP1}$ by IFIXα1. (**a**) Increased $p21^{CIP1}$ protein levels in MDA-MB-468 and MCF-7 IFIXα1 stable cells. Cell lysates isolated from 468-X-1, 468-X-2, MCF-X-1, MCF-X-2, and the control parental (P) and empty vector (V) cell lines were analysed by Western blot using an anti-$p21^{CIP1}$ antibody. Actin served as the loading control. (**b**) Increased $p21^{CIP1}$ mRNA levels in MDA-MB-468 and MCF-7 IFIXα1 stable cells. Total RNA (20 μg) isolated from 468-X-2, MCF-X-2, and the corresponding empty vector (V) control cell lines were analysed by Northern blot using an IFIXα1 or $p21^{CIP1}$ probe as indicated. The 18S and 28S rRNA bands on the membrane after transfer stained by ethidium bromide serve as loading control. (**c**) Inhibition of the kinase activity of Cdk2 and $p34^{Cdc2}$ by IFIXα1. Cell lysates isolated from 468-X-2, MCF-X-2, and the parental (P) control cell lines were immunoprecipitated by Cdk2 (or $p34^{Cdc2}$)-specific antibody followed by Histone H1 (H1) kinase assay. Immunoprecipitation followed by Western blot (IP/W) with Cdk2 or $p34^{Cdc2}$ antibody served as the loading control. (**d**) IFIX expression affects cell cycle distribution. The IFIX-expressing cells (X-2) and the empty vector control cells (V) derived from MCF-7 and MDA-MB-468 cells were subjected to flow cytometry analysis. The percentage of each cell line in G1, S, and G2/M phases was calculated. This result was obtained from two independent experiments

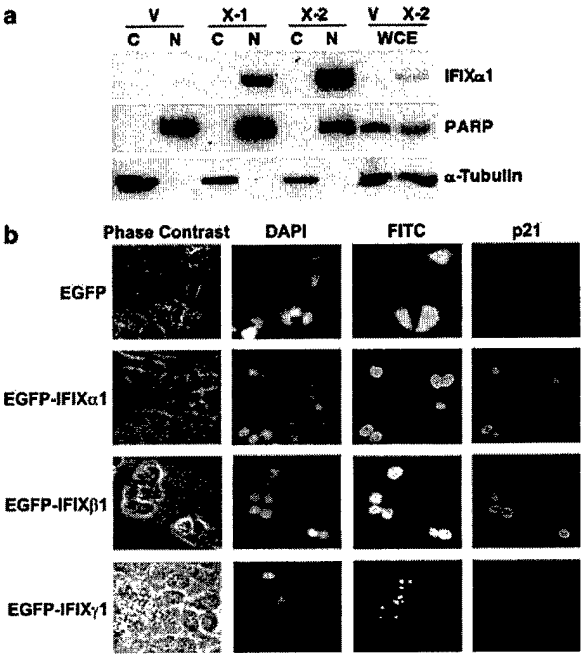


Figure 8 IFIX proteins are localized in the nucleus. (**a**) The stably transfected IFIXα1 protein is localized in the nucleus. Cytoplasmic (C), nuclear (N), or whole cell extracts (WCE) isolated from MCF-X-1, MCF-X-2, or the MCF-7 empty vector control cells (V) were analysed for IFIXα1 expression by Western blot using an anti-Flag antibody. The same blot was used to verify the quality of the extracts using the antibodies against the nuclear protein, PARP, and the cytoplasmic protein, α-Tubulin. (**b**) The transiently transfected IFIXα1 protein is localized in the nucleus. MCF-7 cells were transfected with the plasmid encoding EGFP-tagged IFIXα1, β1, or γ1 protein. The EGFP-expression vector serves as a control. Phase contrast, nuclear staining (DAPI), green fluorescence (FITC), and Texas Red for $p21^{CIP1}$ staining (p21) of each transfection are shown. At 48 h after transfection, the percentage of $p21^{CIP1}$-positive in EGFP-positive cells was counted for each transfection: EGFP (0.95%, 1/105), IFIXα1 (64%, 68/107), IFIXβ1 (52%, 55/106), and IFIXγ1 (2%, 2/100). Cells were examined at × 60 magnification

200-amino-acid domain may be responsible for the upregulation of $p21^{CIP1}$.

Discussion

We identified IFIX as a new member of the human HIN-200 protein family. At least six isoforms were identified in Daudi cells. Interestingly, while the 'form 1' isoforms are present in IFIX-expressing cells such as MCF-10A, MCF-12A, MDA-MB-231, and MDA-MB-435, the 'form 2' isoforms were either missing or undetectable in these cells. A systematic analysis on the expression of 'form 2' isoforms is required to determined whether 'form 2' expression is specific in hematopoietic cells and/or caused by IFN treatment. Using a commercial cDNA expression panel, we found that IFIXα was detectable in only two out of 12 breast carcinomas, whereas most (10 out of 12) of the normal breast tissues have detectable IFIXα expression (Figure 4a). This result was consistent with that of five pairs of matched normal versus tumor samples we collected from patients in which IFIXα expression is downregulated in all tumor samples as compared with that of the matched normal breast tissue samples (Figure 4b). Furthermore, seven out of nine breast cancer cell lines examined in this study have no detectable IFIX, while IFIX expression is readily detectable in the nontransformed breast cell lines (Figure 4c). Although it is possible that the signals on the northern blots may come from other HIN-200 genes due to sequence homology, we consider the IFIXα1 cDNA probe used in these experiments is quite specific

based on the size and the tissue distribution patterns of IFIX mRNA. First, the size of IFIX mRNA (including all isoforms) is ~2.4 kb (between 28S and 18S) (Figure 3a and b), which would effectively rule out the HIN-200 genes MNDA (2.0 kb, at 18S) and AIM2 (1.8 kb, below 18S) (De Young *et al.*, 1997). However, the size of IFI16 mRNA is ~2.7 kb, it is possible that it may migrate closely with IFIX mRNA. Second, in contrast to the expression of IFI16 mRNA in lymphoid tissues such as spleen, thymus, and peripheral blood leukocyte, and many other organs (Wei *et al.*, 2003), the expression of IFIX mRNA is clearly restricted to the secondary lymphoid organs such as spleen, lymph nodes, and peripheral blood leukocyte but not the primary lymphoid organs such as thymus and bone marrow (Figure 3b). Thus, based on the size and tissue distribution patterns of different HIN-200 mRNAs, we believe that, under our experimental conditions, the signals in the Northern blots are mainly generated from the probe hybridizing with IFIX mRNA. Albeit, we cannot rule out the possibility that the additional weak band detected above the IFIX signals in Figure 3b is IFI16 mRNA based on its slightly higher molecular weight and its expression in the thymus. The IFIX-specific antibodies are being generated to detect IFIX proteins by Western blot and immunostaining. Once these critical reagents are available, a more systematic analysis of IFIX protein expression on the breast and normal tumor tissues will be performed to confirm the RT–PCR and Northern blot data presented in this study, and to further investigate the diagnostic and prognostic values of IFIX expression in breast cancer. To understand how IFIX is downregulated in certain breast tumors or breast cancer cell lines but not in others (Figure 4a and c), further genetic and biochemical analysis is necessary.

The expression of HIN-200 was originally identified in hematopoietic cells and was thought to be restricted in this cell type (Dawson and Trapani, 1996). However, recent reports have shown that IFI16 is expressed in epithelial cells in addition to lymphoid cells (Gariglio *et al.*, 2002; Wei *et al.*, 2003). Our finding that IFIX expresses in normal breast tissues (Figure 4a and b) and nontransformed breast epithelial cell lines (Figure 4c) supports the notion that HIN-200 expression is not restricted in hematopoietic cells. Taken together, these observations suggest that IFIX may play a role in maintaining the normal growth of epithelial cells and the downregulation of IFIX expression may contribute to the uncontrolled cell growth and leads to tumorigenesis. It is intriguing that the two breast cancer cell lines that express IFIX, that is, MDA-MB-435 and MDA-MB-231, are metastatic in experimental systems (Price *et al.*, 1990; Zhang *et al.*, 1991). This observation raises a possible link between IFIX expression and metastatic potential of breast cancer cells. However, this possibility is not supported by the available information. (The manufacturer did not provide the metastasis status of patient samples used in Figure 4a.) Of the five breast tumors we collected from patients, all expressed reduced levels of IFIX (Figure 4b), including two recurrent metastatic breast tumors in the chest wall (#1 and #2) and three primary breast tumors (#3, #4, and #5). Moreover, except the BT-474 cell line which was isolated from a solid invasive ductal carcinoma, all breast cancer cell lines used for Northern blot analysis shown in Figure 4c were isolated from metastatic breast tumors: MCF-7, T47-D, MDA-MB-435, MDA-MB-231, and MDA-MB-468 (pleural effusion); MDA-MB-361 (brain metastasis); MDA-MB-453 (effusion); and ZR-75-1 (ascitic effusion). Thus, there is no clear correlation between the expression of IFIX and the metastatic potential of breast cancer in human patients.

Compared to the control MDA-MB-468 cells (P and V), MDA-MB-468 derivatives that expressed exogenous IFIXα1 formed fewer colonies in soft agar and resulted in tumors that grew slower in mice, suggesting that IFIXα1 suppresses tumorigenicity (Figure 5d and e). However, the differences between IFIXα1-expressing derivatives and their parental cells could be due to clonal difference. Moreover, although stably expressing IFIXα1 in breast cancer cell lines are appropriate for proof-of-principle experiments, it is inappropriate for predicting treatment outcome in patients. We therefore performed an IFIXα1-based gene therapy to determine if it would yield efficacy in an orthotopic breast cancer xenograft model. As shown in Figure 6, direct injection of IFIXα1 complexed with the liposome SN2 into tumors yielded a significant antitumor activity as compared to the empty vector control. This result supports the notion that the reduced tumorigenicity of 468-X-2 (Figure 5e) is caused by IFIXα1 expression and not by clonal difference. Importantly, it clearly demonstrates a feasibility of using IFIXα1 as a potential antitumor agent. Since breast cancer is a metastatic disease, this observation should set the stage for testing the therapeutic efficacy of IFIXα1 in systemic treatments delivered by either SN2 liposome (Zou *et al.*, 2002) or viral vectors (Ding *et al.*, 2002).

IFN has been shown to increase the expression of $p21^{CIP1}$ and this is critical for IFN to suppress the anchorage-independent growth of breast cancer cells (Gooch *et al.*, 2000). Consistent to that observation, the expression of IFIXα1, an IFN-inducible protein (Figure 3a and 5a), reduces the growth of breast cancer cells in soft agar (Figure 5d) and increases the expression of $p21^{CIP1}$ (Figure 7a and b). This observation suggests that IFIXα1 may mediate $p21^{CIP1}$ upregulation in response to IFN. The result that IFIXα1 is able to upregulate $p21^{CIP1}$ in MDA-MB-468 cells, which express only mutant p53, indicates the upregulation of $p21^{CIP1}$ by IFIXα1 is independent of p53. The p53-independent upregulation of $p21^{CIP1}$ has been well documented (Cox, 1997; Nadal *et al.*, 1997; Fang *et al.*, 2000; Hingorani *et al.*, 2000). In particular, our observation is reminiscent of a previous finding that overexpression of a mouse HIN-200 protein, that is, p202*a*, also resulted in a p53-independent upregulation of $p21^{CIP1}$ (Gutterman and Choubey, 1999). Since the regulation of $p21^{CIP1}$ expression could take place on transcriptional and/or posttranscriptional levels (Funk and Galloway, 1998; Dotto, 2000), further determination of the half-life of $p21^{CIP1}$ mRNA and

protein, and the effect on the $p21^{CIP1}$ transcriptional activity by IFIX will be necessary to elucidate the mechanism underlying the IFIX-mediated $p21^{CIP1}$ upregulation. As expected, the upregulation of $p21^{CIP1}$ leads to hypo-phosphorylation of pRB in IFIX-expressing MCF-7 cells (data not shown). However, since MDA-MB-468 cells lack pRB (Yin *et al.*, 2001), the inhibition of E2F/pRB pathway by $p21^{CIP1}$ cannot account for the mechanism for IFIX-mediated growth inhibition of MDA-MB-468 cells. Our finding that the kinase activity of $p34^{Cdc2}$ is reduced in IFIXα1-expressing MDA-MB-468 cells (Figure 7c) suggests that IFIXα1 may suppress the growth of MDA-MB-468 cells through the inhibition of $p34^{Cdc2}$ kinase activity at the G2/M phase of the cell cycle by $p21^{CIP1}$. This interpretation is further supported by the results of flow cytometry analysis (Figure 7d) that show G1 arrest in IFIXα1-expressing MCF-7 cells in which pRB/E2F pathway is intact because $p21^{CIP1}$ upregulation leads to inactivation of Cdk2 and the subsequent activation of pRB, resulting in a blockage at the G1/S-phase transition (Dotto, 2000). In the case of IFIXα1-expressing MDA-MB-468 cells, although Cdk2 is inactivated, cells progress through G1/S-phase because pRB/E2F pathway is absent. However, the upregulation of $p21^{CIP1}$ also leads to inactivation of $p34^{Cdc2}$, a critical Cdk controlling G2/M transition (Doree and Hunt, 2002), resulting in S- and G2/M-phase accumulation.

Although it has been shown that two copies of the 200-amino-acid motif of a mouse HIN-200 protein are required for growth inhibition (Gribaudo *et al.*, 1999), a more recent study showed that the overexpression of a human HIN-200 protein, AIM2, which has only one copy of the type *a* 200-amino-acid motif, is sufficient to suppress cell growth (Choubey *et al.*, 2000). Thus, our findings support the notion that, at least in human HIN 200 proteins, a single 200-amino-acid motif is sufficient for growth suppression. Interestingly, IFIXγ does not have the characteristic 200-amino-acid signature motif of the HIN-200 family proteins (Figure 1a and 2d) and forms a speckled nuclear pattern (Figure 8b). Thus, it is likely that IFIXγ plays a distinct functional role from α and β isoforms. Indeed, we showed that, while IFIXα1 or IFIXβ1 induced $p21^{CIP1}$ expression, IFIXγ1 did not (Figure 8b). This result also suggests the 200-amino-acid signature motif may be required for $p21^{CIP1}$ upregulation and, possibly, growth suppression. Given that the pyrin domains are known to be involved in protein–protein interactions (Fairbrother *et al.*, 2001; Martinon *et al.*, 2001; Staub *et al.*, 2001), it is possible that IFIXγ may function as a dominant-negative protein by interacting with IFIXα/β or other pyrin domain-containing proteins.

In summary, IFIX, a newly identified HIN-200 gene, is downregulated in breast cancer. The data presented here indicate that IFIXα1 expression is associated with growth retardation, loss of tumorigenicity, and $p21^{CIP1}$ upregulation in breast cancer. Moreover, efficacy of an IFIXα1-based gene therapy is demonstrated, raising the possibility of using IFIXα1 as a therapeutic agent in breast cancer treatment.

Materials and methods

Cell lines and plasmids

MCF-10A and MCF-12A cells were maintained in DMEM/F12 media containing 5% horse serum, 10 μg/ml bovine insulin, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, and 250 ng/ml fungizone. Daudi, Raji, HL-60, and U937 cells were grown in RPMI medium containing 10% fetal bovine serum. All other cell lines were cultured in DMEM/F12 media containing 10% fetal bovine serum. The IFIXα1 expression vector CMV-IFIXα1 was constructed by inserting IFIXα1 cDNA into pCMV-Tag2B (Flag) (Stratagene, La Jolla, CA, USA). To generate IFIXα1 stable cell lines, CMV-IFIXα1 was transfected into MDA-MB-468 or MCF-7 cells. After 3 weeks of G418 selection (500 μg/ml), the G418-resistant colonies were screened for IFIXα1 expression by Western blot using an anti-Flag antibody (M5, Sigma, St Louis, MO, USA). Control derivatives of MDA-MB-468 and MCF-7 that carry pCMV-Tag2B were similarly established. The EGFP-IFIX expression vectors were constructed by inserting IFIXα1, β1, and γ1 cDNAs into pEGFP-C vectors (BD Biosciences).

Identification and cloning IFIX gene

To identify a new human HIN-200 gene that might be the ortholog of mouse p202, we first used the amino-acid sequence of p202 to query human specific nr, est, and htgs database in the National Center for Biotechnology Information (NCBI) using the tblstn protocol and identified a potential new gene located between MNDA and IFI16 at chromosome 1. As the predicted amino-acid sequence of the new gene appeared to be more homologous to human HIN-200 members than to mouse p202, we then used the IFI16 amino-acid sequence to query these same databases using tblastn. This query allowed us to extend the length of this new gene. Primers were then designed according to the sequences of this potential gene to isolate cDNA by RT–PCR.

Determination of gene expression

The expression of IFIX in cell lines was determined by using Northern blot analysis performed as previously described (Wen *et al.*, 2001). The expression of GAPDH was used as a control for RNA loading. Human cDNAs derived from 12 normal breast tissues (normal) and 12 breast carcinoma tissues (tumor) (Human Breast Cancer Rapid-Scan™ Gene Expression Panel, Origene Technologies, Inc. Rockville, MD, USA) were analysed for IFIX expression by PCR using primers specific to IFIXα under the condition suggested by the manufacturer. The IFIXα1 cDNA was used as a DNA template for the IFIXα-specific positive control. β-actin specific primers (provided by the manufacturer) were used to amplify the β-actin-specific band as an internal control. We used RT–PCR to determine the expression of IFIXα in normal and cancerous breast tissues from five patients with various stages of breast cancer including one with only ductal carcinoma *in situ*. Total RNA was isolated from tissues using Atlas Pure Total RNA Labeling System (BD Biosciences) and reverse transcription was performed using SuperScript First-strand Synthesis System (Invitrogen, Carlsbad, CA, USA). PCR was performed for 35 cycles at 94°C for 40 s, 56°C for 1 min, and 72°C for 40 s. Primers 5′-GGAACAGAGTCAG CATCC-3′ (exon 7) and 5′-CTGCTGGATGGCGGTTGG-3′ (exon 8) were used to amplified a 224 bp fragment specific to IFIXα (Figure 4a and b).

In addition, to detect both IFIXα (265 bp) and β (140 bp) isoforms in cell lines (Figure 4d), the following primers are used: 5′-GGAACAGAGTCAGCATCC-3′ (exon 7) and 5′-GTTATTTGATATCCTTGTCC-3′ (exon 9). To detect IFIXγ isoforms (γ1, 744 bp and γ2, 717 bp), the following primers are used: 5′-TTAGAGATGGCAAATAACTAC-3′ (exon 2) and 5′-TTAGTGAGCAAAGGGAATG-3′ (exon 4′). To detect the expression of the 'form 2' isoforms ('form 1', 161 bp and 'form 2', 134 bp), the following primers are used: 5′-TTGGGC AAACTAATAGAATTC-3′ (exon 2) and 5′-GCAGGATA CACTTCTTTCTG-3′ (exon 3). As a control for the quality of the RNA samples, an ~600 bp GAPDH cDNA fragment was amplified using primers 5′-TGAAGGTCGGAGTCAACG GA-3′ and 5′-GGCATGGACTGTGGTCATGA-3′. To detect all IFIX isoforms (~350 bp) (Figure 5a, bottom panel), the following primers are used: 5′-TGATGGAGGAAAAGTT CC-3′ (exon 2) and 5′-TGCTGGCTCCTGCAGAGC-3′ (exon 4).

Determination of growth, in vitro *transformation and* in vivo *tumorigenicity of breast cancer cells*

MTT and soft agar assays were used to determine the anchorage-dependent and -independent *in vitro* cell growth, respectively, and were performed as previously described (Shao, 1997). To measure the effect of IFN-γ on cell growth (Gooch *et al*., 2000), MCF-7 and MDA-MB-468 cells were planted in 24-well plates (18,000 cells/well) in DMEM/F12 media supplemented with 10% fetal calf serum. The next day, cells were washed in 1 × PBS and grown in serum-free DMEM/F12 media overnight. The serum-free media was replaced with DMEM/F12 media containing 0.25% fetal calf serum. IFN-γ (1000 U/ml) was added. The growth of the cells was measured by MTT assay at 48 h. To determine tumorigenicity, 1×10^6 cells were injected into the MFP of 6-week-old female nude mice and the growth of tumors was monitored weekly. For each experiment, a cell line was injected into three mice with each mouse injected at 2 MFP.

IFIX α1 gene therapy

One million MDA-MB-468 cells in 200 μl of PBS were injected into a MFP of 4–5-week old female nude mice. Each cell line was injected into 5 mice with each mouse injected at 2 MFP. After the tumors grew to 0.5 cm in diameter, mice were treated twice a week by intratumoral injection. Tumor-bearing mice were randomly divided into two equal treatment groups with each tumor injected with 22.5 μl of the liposome SN2 in 50 μl of PBS (Zou *et al*., 2002) mixed with 15 μg of either CMV-IFIXα1 or a control vector pCMV-Tag2B (100 μl total injection volume).

Histone H1 kinase assay

Cells were lysed with RIPA-B buffer (20 mM Na_2PO_4 (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM Na_3VO_4, 5 mM phenylmethylsulfonyl fluoride, 1% aprotinin). Lysate containing 200–400 μg of protein was incubated at 4°C for 1 h with 2 μg of anti-Cdk2 antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) or 1.5 μg anti-$p34^{Cdc2}$ antibody (Santa Cruz Biotech.), followed by incubation with Protein A-agarose for 2 h. The immunoprecipitates were washed twice with PBS, once with kinase buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, and 0.5 mM dithiothreitol), and then resuspended in 40 μl of kinase buffer containing 2 μg of histone H1 (Sigma), 25 μM ATP, and 5 μM γ-^{32}P ATP. The kinase reaction was terminated by adding 40 μl of SDS–PAGE loading buffer after a 15 min incubation at room temperature (Cdk2) or 30 min incubation at 30°C ($p34^{Cdc2}$). Samples were resolved by SDS–PAGE and the phosphorylated Histone H1 was visualized by autoradiography.

Western blot

The standard procedure has been described previously (Wen *et al*., 2001). The antibodies used in this study are anti-Flag (Sigma, M5), anti-β-actin (Sigma), anti-$p21^{CIP1}$ (Santa Cruz Biotech.), anti-poly-(ADP-ribose) polymerase (PARP) (BD Biosciences), and anti-α-Tubulin (Sigma). The cytoplasmic and nuclear extracts were isolated according to the protocol described previously (Xie and Hung, 1994).

Immunostaining

MCF-7 cells (1×10^4 in 0.5 ml). were cultured in a four-well glass chamber overnight. Cells were then transfected with 1 μg of the plasmid encoding EGFP-tagged IFIXα1, β1, or γ1 fusion protein. The EGFP expression vector serves as a control. At 48 h after transfection, cells were washed with PBS and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature followed by PBS wash. The primary $p21^{CIP1}$ monoclonal antibody (Santa Cruz Biotech.) (1 : 100) was incubated with the cells at 37°C for 1 h. Cells were then washed with PBS, followed by incubation with the rabbit anti-mouse secondary antibody conjugated with Texas Red (1 : 200) at 37°C for 45 min. After incubation, cells were washed briefly with PBS and air-dried, followed by incubation with the blue fluorescent dye DAPI (1 : 100 in 50% glycerol/PBS). A cover slip was placed on top of the slide for visualization by microscopy.

GenBank Accession numbers

IFIXα1 (AY185344), IFIXα2 (AY185345), IFIXβ1 (AY185346), IFIXβ2 (AY185347), and IFIXγ1 (XM086611).

Note Added In Proof

During the preparation of this manuscript, a partial amino acid sequence of the N-terminal domain of IFIX protein (referred to as IFI16-like protein 1) was published (Liepinsh *et al*., 2003).

Acknowledgements
We thank Drs Mien-Chie Hung, Funda Meric, Naoto Ueno, Dihua Yu, Mong-Hong Lee, and Nancy Poindexter for providing the reagents used in this study. This work was supported in part by grants from the Department of Defense (DAMD17- 99-1-9270), Texas Advanced Technology Program (003657-0082-1999), and an Institutional Research Grant from The University of Texas, MD Anderson Cancer Center (to DHY), and Cancer Center Core Grant CA16672 from the NIH. YD is the recipient of a post-doctoral fellowship from the Department of Defense (DAMD17-02-1-0451).

References

Choubey D. (2000). *J. Biol. Regul. Homeost. Agents*, **14,** 187–192.
Choubey D, Walter S, Geng Y and Xin H. (2000). *FEBS Lett.*, **474,** 38–42.
Coradini D, Pellizzaro C, Biffi A, Lombardi L, Pirronello E, Riva L and Di Fronzo G. (1998). *Anticancer Res.*, **18,** 177–182.
Cox LS. (1997). *J. Pathol.*, **183,** 134–140.
Dawson MJ and Trapani JA. (1995). *J. Cell. Biochem.*, **57,** 39–51.
Dawson MJ and Trapani JA. (1996). *J. Leukotr. Biol.*, **60,** 310–316.
De Young KL, Ray ME, Su YA, Anzick SL, Johnstone RW, Trapani JA, Melzer PS and Trent JM. (1997). *Oncogene*, **15,** 453–457.
Ding Y, Wen Y, Spohn B, Wang L, Xia W, Kwong KY, Shao R, Li Z, Hortobagyi GN, Hung M-C and Yan D-H. (2002). *Clin. Cancer Res.*, **8,** 3290–3297.
Doree M and Hunt T. (2002). *J. Cell Sci.*, **115,** 2461–2464.
Dotto GP. (2000). *Biochim. Biophys. Acta*, **1471,** M43–M56.
Fairbrother WJ, Gordon NC, Humke EW, O'Rourke KM, Starovasnik MA, Yin JP and Dixit VM. (2001). *Protein Sci.*, **10,** 1911–1918.
Fang M, Liu B, Schmidt M, Lu Y, Mendelsohn J and Fan Z. (2000). *Anticancer Res.*, **20,** 103–111.
Funk JO and Galloway DA. (1998). *Trends Biochem. Sci.*, **23,** 337–341.
Gariglio M, Azzimonti B, Pagano M, Palestro G, De Andrea M, Valente G, Voglino G, Navino L and Landolfo S. (2002). *J. Interferon Cytokine Res.*, **22,** 815–821.
Gooch JL, Herrera RE and Yee D. (2000). *Cell Growth Differ.*, **11,** 335–342.
Gribaudo G, Riera L, De Andrea M and Landolfo S. (1999). *FEBS Lett.*, **456,** 31–36.
Gutterman JU and Choubey D. (1999). *Cell Growth Differ.*, **10,** 93–100.
Hingorani R, Bi B, Dao T, Bae Y, Matsuzawa A and Crispe IN. (2000). *J. Immunol.*, **164,** 4032–4036.
Johnstone RW and Trapani JA. (1999). *Mol. Cell. Biol.*, **19,** 5833–5838.
Kimchi A, Resnitzky D, Ber R and Gat G. (1988). *Mol. Cell. Biol.*, **8,** 2828–2836.
Lengyel P. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 5893–5895.
Liepinsh E, Barbals R, Dahl E, Sharipo A, Staub E and Otting G. (2003). *J. Mol. Biol.*, **332,** 1155–1163.
MacLachlan TK, Sang N and Giordano A. (1995). *Crit. Rev. Eukaryot. Gene Express.*, **5,** 127–156.
Martinon F, Hofmann K and Tschopp J. (2001). *Curr. Biol.*, **11,** R118–R120.
Nadal A, Jares P, Cazorla M, Fernandez PL, Sanjuan X, Hernandez L, Pinyol M, Aldea M, Mallofre C, Muntane J, Traserra J, Campo E and Cardesa A. (1997). *J. Pathol.*, **183,** 156–163.
Naldini A, Carney DH, Pucci A and Carraro F. (2002). *J. Cell. Physiol.*, **191,** 290–297.
Price JE, Polyzos A, Zhang RD and Daniels LM. (1990). *Cancer Res.*, **50,** 717–721.
Shao R, Karunagaran D, Zhou BP, Li K, Lo S-S, Deng J, Chiao P and Hung M-C. (1997). *J. Biol. Chem.*, **272,** 32739–32742.
Staub E, Dahl E and Rosenthal A. (2001). *Trends Biochem. Sci.*, **26,** 83–85.
Wei W, Clarke CJP, Somers GR, Cresswell KS, Loveland KA, Trapani JA and Johnstone RW. (2003). *Histochem. Cell. Biol.*, **119,** 45–54.
Wen Y, Yan D-H, Wang B, Spohn B, Ding Y, Shao R, Zhou Y, Xie K and Hung M-C. (2001). *Cancer Res.*, **61,** 7142–7147.
Wen Y, Yan DH, Spohn B, Deng J, Lin SY and Hung MC. (2000). *Cancer Res.*, **60,** 42–46.
Xie Y and Hung MC. (1994). *Biochem. Biophys. Res. Commun.*, **203,** 1589–1598.
Yin F, Giuliano AE, Law RE and Van Herle AJ. (2001). *Anticancer Res.*, **21,** 413–420.
Yu D, Jing T, Liu B, Yao J, Tan M, McDonnell TJ and Hung MC. (1998). *Mol. Cell*, **2,** 581–591.
Zhang JF, Hu C, Geng Y, Selm J, Klein SB, Orazi A and Taylor MW. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 4513–4518.
Zhang RD, Fidler IJ and Price JE. (1991). *Invasion Metastasis*, **11,** 204–215.
Zhou Y, Wang S, Yue BG, Gobl A and Oberg K. (2002). *Cancer Investig.*, **20,** 348–356.
Zou Y, Peng H, Zhou B, Wen Y, Wang SC, Tsai EM and Hung MC. (2002). *Cancer Res.*, **62,** 8–12.